REMARKS

Status of the Claims

Claims 1-11 are pending. Claims 2, 3, and 7-11 have been withdrawn from further consideration by the Examiner as being drawn to a non-elected invention. Claims 1 and 4-6 are currently under consideration. Claims 1, 4, 5, and 6 have been amended herein to more particularly point out the invention. Support for these amendments is found in the specification at least on page 1, lines 12-14 and 17-18; page 4, lines 31-36; page 5, lines 16-17; page 6, lines 12-28; page 15, lines 34-36; and page 16, line 1-page 17, line 19. Support for new claim 12 is found in the specification on page page 8, lines 1-9; page 40, lines 5-26. No new matter has been added.

Indefiniteness Rejection Under 35 U.S.C. § 112

Claims 1 and 4-6 stand rejected under 35 U.S.C. § 112 second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Office Action states that claims 1 and 4-6 are indefinite because it is unclear how to measure what is an effective amount of a "combination therapy." (Page 31, ¶ 5.) Applicants submit that the amendments to claim 1 have obviated the rejection. Claim 1 now recites "a gene therapy vector encoding a lysosomal hydrolase," "an exogenously produced natural or recombinant enzyme," and "a small molecule." Applicants submit that a skilled artisan could readily measure an effective amount of any combination of these elements, especially in light of the specification's teachings. (*See e.g.* page 2, lines 10-21; page 23, lines 6-23; and page 17, lines 16-31.)

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The Office Action further state that claims 1 and 4-6 are indefinite because they omit essential steps. (Page 3, ¶ 4.) The Office alleges the omitted steps include: what is being administered in the combination therapy of gene therapy and enzyme replacement therapy and whether that combination therapy ameliorates the symptoms of Fabry disease. Without conceding the correctness of the rejection and for the sole purpose of expediting prosecution, Applicants have amended claims 1, 4 and 6 to obviate the rejection. Claim 5 depends on claim 1 and, thus, the amendment to claim 1 obviates the rejection with respect to claim 5 as well.

Enablement Rejection Under 35 U.S.C. § 112

Claims 1 and 4-6 stand rejected under 35 U.S.C. § 112 first paragraph as allegedly not enabled by the specification. The Office alleges that claims 1 and 4-6 are not enabled because the specification fails to provide adequate guidance and evidence regarding how to use any gene for gene therapy and any protein for enzyme replacement therapy. While admitting that progress has been made in recent years in the field of gene transfer in vivo, the Office, nonetheless, alleges that vector targeting to desired tissues in vivo continues to be unpredictable and inefficient. The Office concludes it would require undue experimentation for one skilled in the art to practice the full scope of the claimed invention. (Page 3-6, ¶ 7) For the reasons set forth below, Applicants submit that this conclusion is incorrect.

A. The Standard For Enablement

"The test for enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known

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in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 U.S.P.Q.2d 1217, 1223 (Fed. Cir. 1988). "A patent need not teach and preferably omits what is well known in the art." *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991); MPEP § 2164.01. The Office bears the initial burden in establishing a reasonable basis to question enablement. MPEP §2164.04.

B. The Claimed Invention Is Enabled

Applicants have amended claims 1 and 4 to recite a "lysosomal hydrolase," thus obviating the Office's concern regarding the use of any gene or any enzyme. The claims also recite "a method of treating a subject diagnosed as having Fabry disease." The Office contends that because no in vivo data exists, the invention is not enabled. Applicants disagree.

There is a well-established, art recognized, animal model for Fabry disease. *See, e.g.,* Oshima et al., 1997, *Proc Natl Acad Sci USA,* 94:2540; U.S. Patent 6,066,626 (courtesy copies enclosed). Applicants note that U.S. Patent 6,066,626 is cited and incorporated by reference in the specification at page 23, lines 27-29, and page 40, lines 34-36. Both Oshima et al. and U.S. Patent 6,066,626 describe a knockout mouse that lacks α galactosidase A activity, resulting in the accumulation GL3 over time. Moreover, the same mouse is described in Ioannou, cited by the Examiner. It is well established that the patho-physiology of this knock-out mouse is similar to the patho-physiology associated with Fabry disease. Moreover, U.S. Patent 6,066,626 demonstrates that gene therapy can restore α galactosidase A activity and decrease the accumulation of GL3. The art thus provides an accepted system to test the claimed combinations. The Office has not presented any evidence to suggest that a correlation

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between data obtained using this animal model does not correlate with in vivo results. See MPEP § 2164.02

Moreover, the instant specification teaches at page 23, lines 6-12 a specific test which can monitor whether a specific combination therapy is effective at treating for Fabry disease, including assays which monitor α galactosidase A activity and GL3 accumulation. These assays were well known in the art. See, U.S. Patent 6,066,626 at column 13, line 14-column 14, line 58. Using only routine experimentation, the skilled artisan could readily test any of the combinations recited in the claims using the knock-out mouse model and known assays, disclosed in the specification, for effective therapies. Given that both the assays and the mouse model were known in the art, and disclosed in the specification, and that U.S. Patent 6,066,626 teaches how to make and use viral and non-viral vectors for treating Fabry disease, such testing would be merely routine in light of *In re Wands*, 858 F.2d 731, 8, U.S.P.Q. 2d 1400. Accordingly, Applicants submit that claims 1 and 4-6 are enabled by the specification.

C. The Cited References

The Office alleges that gene therapy is unpredictable and thus, not enabled, citing to Deonarain, 1998, *Expert Opin. Ther. Patents* 8:53; Verma et al. 1997; *Nature*: 389239; Eck et al., 1996 *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, p. 77-101(McGraw Hill, New York); and Gorecki, 2001, *Expert Opin. Emerging Drugs* 6(2):187 in support of this position and thus, not enabled. Applicants respectfully submit that the cited references do not support this conclusion.

The Office quotes the second sentence from Deonarain, which discusses one obstacle to gene therapy. The very next sentence, however, states: "viral methods for

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gene delivery have been studied for a number of years and are effective vectors for gene transfer." The reference, when considered in its entirety, does not express concern for the unpredictability of gene therapy, but rather lauds a new improved approach to gene therapy (ligand-targeted receptor mediated endocytosis). Applicants' submit that the Office has merely taken one sentence out of context to imply a conclusion (*i.e.* all gene therapy is unpredictable) that was never intended by the author.

Although, Verma does discuss problems associated with viral vectors used in gene therapy, it does not suggest any problems associated with non-viral vectors. Moreover, even with respect to problems associated with viral vectors, Verma notes on page 241, that AAV overcomes many of these problems and has been successfully used in a mouse model for hemophilia. Moreover, Verma was published in 1997 and does not necessarily reflect the state of the art at the effective filing date of this application (*i.e.,* June 2000). The same is true with respect to Eck which was published in 1996.

The Office quotes the abstract of Gorecki as stating obstacles to gene therapy in vivo include "the development of effective clinical products," and "the low levels and stability of expression and immune responses to vectors and/or gene products." Applicants submit that the Office has again taken a single statement out of context and mischaracterized the impact of the entire reference. Gorecki also states "The progress in AAV and lentivirus vectors, improved regulation of transgene expression, and advances in cell technology are among the recent most exciting developments," (Abstract). Gorecki also notes the first positive results have now been seen in clinical trials (page 187). Accordingly, when considered in its entirety, Gorecki does not

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disclose that gene therapy is unpredictable, as the Office suggests, but rather that a technology that holds great promise has begun to bear fruit. More importantly, as Applicants have previously noted, gene replacement therapy has been shown to be effective in a mouse model for Fabry disease (U.S. Patent 6,066,626).

Accordingly, for the reasons put forth above, Applicants respectfully submit that the claimed invention is enabled and thus the rejection 35 U.S.C. § 112 first paragraph should be withdrawn.

The Rejections Under 35 U.S.C. § 103(a)

Claims 1 and 4-6 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Medin et al., 1996, *Proc. Natl. Acad. Sci. USA,* 93:7917 (Medin) in view of Ioannou et al. 1996, *American Journal of Human Genetics,* 59(4)Supp. A15 (Ioannou). The Office alleges that Medin teaches construction of a recombinant retroviral vector expressing a human α galactosidase A and correction of the enzymatic defect in multiple types of cells obtained from patients with Fabry disease. The Office also alleges that Medin teaches the uptake of secreted α galactosidase A from the transduced cells by uncorrected cells. The Office further alleges that Ioannou teaches enzyme replacement therapy in a α galactosidase A deficient mouse. The Office concludes that the claimed invention is obvious in light of these two references. For the reasons stated below this conclusion is incorrect.

The Claimed Invention Is Not Prima Facie Obvious

MPEP § 2143 provides the standard required to establish a prima facie case of obviousness. "First there must be some suggestion or motivation, either in the

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references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine what the reference teaches. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references combined) must teach or suggest all the claim limitations."

The motivation to make the claimed invention and the reasonable expectation of success must both be found in the prior art, not the Applicant's disclosure. *In re Vaeck,* 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991). The references must be considered as a whole and must suggest the desireability, and thus the obviousness of making the combination. *Hodosh v. Block Drug Co., Inc.,* 229 U.S.P.Q. 182, 187 n.5 (Fed. Cir. 1986); MPEP § 2141. The Patent and Trademark Office bears the burden of initially establishing a prima facie case of obviousness. MPEP § 2142. The Office has not met its burden in this case.

No Motivation Existed To Combine The References

Nothing in either Medin or Ioannou would motivate a skilled artisan to combine enzyme replacement therapy with gene therapy. Medin states: "a recombinant retroviral vector has been constructed that engineers efficient transduction of cells and expression of human α-gal A activity. This vector is of high titer, and it corrects the enzymatic defect in multiple types of cells obtained from patients with Fabry disease" (emphasis added) (page 7921, column 1). Medin thus suggests a solution to the problem of treating Fabry disease is at hand.

A skilled artisan reading Ioannou would reach a similar conclusion. Ioannou states: "compared to untreated littermates, the Gl-3 levels in plasma, liver, and heart were markedly decreased, while only a slight decrease was observed in the kidney.

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However, a series of eight injections of the same doses at 48 hour intervals decreased the levels of renal GL-3 by 3--50% . . . . These preclinical results provide the rationale for enzyme replacement in patients with Fabry disease." A skilled artisan would not be motivated to combine Medin and Ioannou because each reference discloses a successful solution to the problem of treating Fabry disease.

Applicants urge the Examiner to consider that the requirement regarding the motivation to combine references has been further clarified in *Winner Int'l Royalty Corp. v. Wang,* 53 U.S.P.Q.2d 1581 (Fed. Cir. 2000.) In *Winner,* the Federal Circuit upheld the district court's determination that the claims at issue were not obvious over two prior art references. *Id.* at 1590. The Court has agreed that there was no motivation to combine these references to arrive at the claimed invention because, in part, "there was no apparent disadvantage to the dead-bolt mechanism of [ref 1], and therefore the motivation to combine would not stem from the 'nature of the problem' facing one or ordinary skill in the art, because no 'problem' was perceived." *Id.* at 1587.

Similarly, in making its rejection of the claimed invention, the Office has not identified any apparent disadvantage of, or problem with, the disclosure of Medin which would have motivated one to combine its teachings with those of Ioannou.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants respectfully request the reconsideration and reexamination of this application and the timely allowance of the pending claims.

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Respectfully submitted,

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α-Galactosidase A deficient mice: A model of Fabry disease

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Contributed by Roscoe O. Brady, December 30, 1996

ABSTRACT **Fabry disease is an X-linked inherited metabolic disorder that is caused by a deficiency of α-galactosidase A (α-Gal A). Progressive deposition of neutral glycosphingolipids that have terminal α-linked galactosyl moieties in vascular endothelial cells causes renal failure along with premature myocardial infarctions and strokes in patients with this condition. No specific treatment is available for patients with this disorder at this time. An animal model of this condition would be valuable for exploring therapeutic strategies for patients with Fabry disease. We report here the generation of *α-Gal A* deficient mice by gene targeting and an analysis of the resulting phenotype. The knockout mice display a complete lack of α-Gal A activity. The mice, however, appeared clinically normal at 10 weeks of age. Ultrastructural analysis revealed concentric lamellar inclusions in the kidneys, and confocal microscopy using a fluorescent-labeled lectin specific for α-D-galactosyl residues showed accumulation of substrate in the kidneys as well as in cultured fibroblasts. Lipid analysis revealed a marked accumulation of ceramidetrihexoside in the liver and the kidneys. These findings indicate the similarity of the pathophysiological process in the mutant mice and in patients with Fabry disease. The deficiency of α-Gal A activity and the accumulation of material containing terminal α-galactosyl residues in cultured embryonic fibroblasts derived from *α-Gal A*(−/0) mice were corrected by transducing these cells with bicistronic multidrug resistance retroviruses containing human *α-Gal A* cDNA.**

Fabry disease is an X-linked inherited disorder of glycolipid metabolism resulting from deficient activity of the lysosomal enzyme, α-galactosidase A (α-Gal A; EC 3.2.1.22) (1). Neutral glycosphingolipids with terminal α-linked galactosyl moieties—globotriaosylceramide [ceramidetrihexoside (CTH): Galα1-4Galβ1-4Glcβ1-1Cer], and galabiosylceramide (Galα1-4Galβ1-1Cer)—accumulate in the liver, heart, spleen, kidney, vascular endothelial cells, and in plasma of the patients with this disorder. Major disease manifestations include paresthesias in the extremities, corneal dystrophy, angiokeratoma, and occlusive vascular disease of the heart, kidney, and brain, leading to premature mortality (for review, see ref. 2). Human *α-Gal A* cDNA (3, 4) and the genomic clone (5) have been isolated and mapped to Xq22 (6). Analysis of the *α-Gal A* gene in Fabry patients revealed heterogeneous molecular lesions such as point mutations and partial gene rearrangements (7).

There is no specific therapy for Fabry disease. Renal transplantation has been performed in Fabry patients with varying outcome (2). Enzyme replacement (8) and somatic gene therapy (9, 10) have potential as effective therapies for lysosomal storage diseases. We have reported efficient expression of the human α-Gal A in NIH 3T3 cells using bicistronic multidrug-resistant (MDR) gene retroviruses (11), and also *in vitro* correction of enzyme deficits in fibroblasts derived from Fabry patients using recombinant retrovirus (12). However, a suitable animal model for Fabry disease is required to evaluate the *ex vivo* and *in vivo* potential of these therapies. Although natural mutant animal models have been reported for other lysosomal disorders such as mucopolysaccharidosis I (13) and VII (14), no animal model has been identified for Fabry disease. An appropriate model of Fabry disease would be invaluable for the study of the molecular pathophysiology of this human genetic disease as well as for the development of effective therapeutic strategies.

To develop a mouse model for Fabry disease by disrupting *α-Gal A* gene by homologous recombination (15), we isolated and characterized the mouse *α-Gal A* gene (16). We found that the mouse and human *α-Gal A* genes are highly similar in size, gene organization, and nucleotide sequence of the coding regions (16). Here we report the generation and characterization of *α-Gal A*-deficient mice and demonstrate the correction of metabolic deficits in the *α-Gal A*-deficient fibroblasts by transducing them with bicistronic MDR retroviruses containing human *α-Gal A* cDNA.

MATERIALS AND METHODS

Gene Targeting and Generation of Mutant Mouse. The targeting construct (TC) contains 7.5 kb of *α-Gal A* genomic sequence (16) in the pPNT vector (17) (Fig. 1*A*). The 1.5 kb of 5′ flanking fragment consisting of *Kpn*I-*Bam*HI fragment, and the 6 kb of *Eco*RI-*Eco*RI fragment (16) were subcloned into *Xho*I site and *Eco*RI site of pPNT, respectively. Tissue culture of J1 embryonic stem (ES) cells (18) and conditions for electroporation of the targeting construct were performed as described (19). Selection with 350 μg/ml G418 (Geneticin, GIBCO) and 2 μM ganciclovir (Syntex, Palo Alto, CA) was started 24 h after electroporation. After 8–10 days, resistant clones were picked, expanded, and analyzed as described (20). Briefly, genomic DNA from these clones was digested with *Eco*RI and hybridized with 5′ flanking probe isolated from the 0.5 kb of *Sac*I-*Kpn*I fragment (Fig. 1*A*). The sizes of the *Eco*RI fragments from wild-type (WT) and mutated (M) allele were 7.5 kb and 8.5 kb, respectively. The presence of a single

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Abbreviations: α-Gal A, α-galactosidase A; MDR, multidrug resistance; FITC, fluorescein isothiocyanate; CTH, ceramidetrihexoside; HPTLC, high-performance thin-layer chromatography; ES cells, embryonic stem cells; IRES, internal ribosome entry site.

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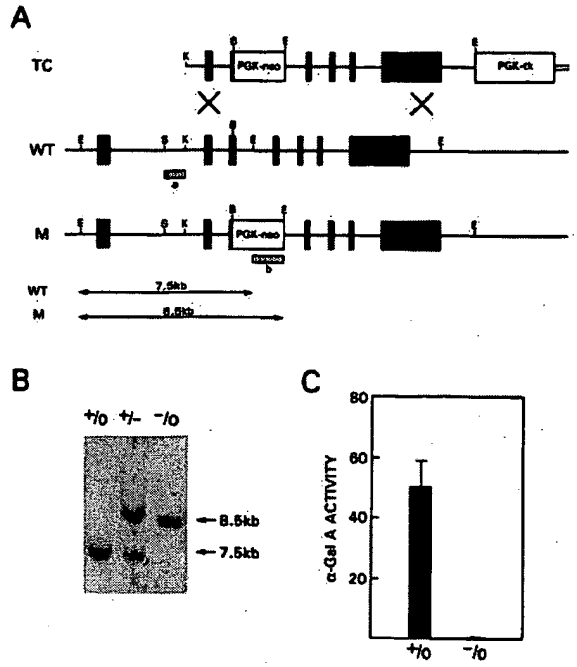

FIG. 1. Targeted disruption of the *α-Gal A* gene in mouse ES cells and generation of *α-Gal A(−/0)* mice. (*A*) Schematic representation of the targeting construct (TC) and the wild-type (WT) and mutated (M) alleles of *α-Gal A* gene. The hatched bars indicate 5′ flanking probe (a) and neomycin resistance gene (neo) probe (b) used to identify targeted clones. Restriction enzyme sites are as follows: K, *Kpn*I; B, *Bam*HI; E, *Eco*RI; S, *Sac*I. (*B*) A representative Southern blot analysis of tail DNA from *α-Gal A(+/0)*, *α-Gal A(+/−)*, and *α-Gal A(−/0)* mice hybridized with probe a. (*C*) α-Gal A activity in liver homogenates from *α-Gal A(+/0)* ($n = 6$) and *α-Gal A(−/0)* ($n = 5$) mice. Activities are expressed as nmol/h per mg protein. Bar = SD.

integration event was confirmed by hybridization with neomycin phosphotransferase gene-specific probe (neo probe) using the same membrane after stripping the 5′ flanking probe (20, 21). Blastocyst injections were performed as described (22). Mice heterozygous for the α-Gal A gene deletion, *α-Gal A(+/−)*, were generated by mating male chimeras with C57BL/6 females. *α-Gal A(−/0)* mutant male mice were generated from matings of *α-Gal A(+/−)* female and C57BL/6 male mice. To determine the genotypes of offspring, tail DNA was digested with *Eco*RI and hybridized with the 5′ flanking probe as described above. Embryonic fibroblasts from mice of each genotype were established using 14.5-16.5 E embryos as described (22).

Histology. For light microscopy, tissues were immersion-fixed for 4 days in 10% phosphate-buffered formalin, and embedded in paraffin, and 10 μm tissue sections were stained with hematoxylin-eosin. For electron microscopy, tissue blocks (<1 mm^3) were fixed in 4% formaldehyde/1% glutaraldehyde in phosphate buffer at 4°C for 24 h. Samples were washed three times in 0.1 M cacodylate buffer with 0.2 M sucrose and postfixed in 1% osmium tetroxide buffered with symcollidine (pH 7.2) at 4°C for 1 h. After dehydration in serial alcohol and propylene oxide, samples were infiltrated with and embedded in Epon substitute. Thin sections were prepared and stained with uranyl acetate and lead citrate. For fluorescence staining with *Griffonia (Bandeiraea) simplicifolia* lectin (GSL1-B_4 isolectin, Vector Laboratories), which is specific for α-D-galactosyl residues (23, 24), fibroblasts cultured on coverslips were fixed with 2% paraformaldehyde in PBS (pH 7.4) for 30 min, followed by permeablization with ice-cold methanol at −20°C for 6 min. Frozen kidney tissues were prepared by embedding in OCT compound, sectioned at 8 μm and allowed to air dry. After incubation with fluorescein isothiocyanate (FITC)-conjugated lectin in TBS (50 mM Tris/150 mM NaCl, pH 7.4) for 1 h at 4°C, slides were washed extensively with TBS. For the inhibition studies, sections were incubated with lectin in the presence of 200 mM D-galactose. Stained cells and tissues were examined with a confocal laser-scanning imaging system (Leica TCS 4-D, Leica, Deerfield, IL) using standard imaging analysis software in a fluorescence mode.

Lysosomal Enzyme Assays. Tissues from each genotype were homogenized with 9 volumes of assay buffer (28 mM citric acid/44 mM dibasic sodium phosphate, pH 4.4) containing 0.5% sodium taurocholate and centrifuged at 14,000 rpm (15,000 × *g*) for 30 min. Cell pellets from cultured fibroblasts were resuspended in 8–10 volumes of the above buffer and sonicated on ice for 5 sec (twice) and then centrifuged as above. The supernatant solutions were assayed for α-Gal A activity by incubation with 5 mM 4-methylumbelliferyl-α-D-galactopyranoside in the presence of 100 mM *N*-acetylgalactosamine (25). This material was added as a specific inhibitor of α-galactosidase B activity. β-Hexosaminidase activity was determined (26) as a control for specificity. Protein concentrations were determined by the method of Lowry *et al.* (27).

Retrovirus Transduction. Two bicistronic retroviruses, Ha-αGal-IRES-MDR and Ha-MDR-IRES-αGal, carrying human *α-Gal A* cDNA and *MDR1* cDNA (11) were used to transduce fibroblasts from the embryos of the wild-type and *α-Gal A* mutant mice. After drug selection with the medium containing 25 ng/ml vincristine (Sigma) for 7–9 days, the resistant clones were isolated, expanded, and analyzed for α-Gal A activity. Clones were also subjected to fluorescence-labeled lectin staining as described above.

Analysis of Glycolipids. Tissues were homogenized in 9 volumes of assay buffer without detergent and extracted with isopropanol and chloroform as described (28). Following partitioning, lipids in the lower phase were dried under a stream of nitrogen and redissolved in a minimum volume of chloroform:methanol (2:1). The mixtures were applied to high-performance thin-layer chromatography (HPTLC) and developed with chloroform:methanol:water (65:25:4). Glycolipids were identified by the characteristic purple color upon spraying with α-naphthol spray reagent (29).

RESULTS

Targeting of the *α-Gal A* Gene. The targeting vector was constructed using the genomic clones of the mouse *α-Gal A* gene isolated from the 129/SvJ mouse genomic library (16). This vector carries a 1-kb deletion spanning part of exon III and intron III. At the site of the deletion, the neomycin resistance gene was inserted as a positive selection marker (Fig. 1*A*). Negative selection against random integration was conferred by a herpes simplex virus thymidine kinase (tk) gene (30). J1 ES cells were electroporated with the targeting vector DNA, and 109 double-resistant clones were isolated and screened by Southern blot analysis (20, 21). Three targeted clones were identified as positive for gene disruption based on the predicted size of the targeted allele. Additional integration of the targeting vector was excluded by hybridization with the neo probe (20, 21). Selected clones were injected into C57BL/6 blastocysts to generate several overt chimeras. Four male chimeras successfully transmitted the *α-Gal A* mutation through the germline (Fig. 1*B*).

To confirm the complete inactivation of the *α-Gal A* gene, we performed enzyme assays using various tissues and embryonic fibroblasts from *α-Gal A* mutant mice and wild-type littermate controls. α-Gal A activity was undetectable in liver homogenates from *α-Gal A(−/0)* mice, whereas it was readily demonstrated in those from wild-type littermates (Fig. 1*C*). Fibroblasts derived from *α-Gal A(-/0)* mice showed negligible α-Gal A activity (Table 1). A similar reduction in α-Gal A

Table 1. α-Gal A activity of fibroblast clones transduced with bicistronic MDR retroviruses containing human *α-Gal A* cDNA

Clone	Genotype	Retrovirus	α-Gal A activity,* nmol/h per mg	Lectin (GSL-B_4) staining†
N1	N	—	527.9	−
N2	N	—	636.0	−
K1	KO	—	1.4	+
K2	KO	—	1.7	+
KM-1	KO	HaMDR	38.8	+
KM-2	KO	HaMDR	4.2	+
KGM-1	KO	Ha-αGal-IRES-MDR	611.0	−
KMG-1	KO	Ha-MDR-IRES-αGal	524.8	−
KMG-2	KO	Ha-MDR-IRES-αGal	274.8	−

Primary fibroblasts from mice of either genotypes were established using 14.5–16.5 E embryos as described (22). Cultured fibroblasts were transduced with indicated retroviruses as described (11). N, normal; KO, knockout.
*Data are expressed as means of three determinations.
†Fluorescence-labeled lectin binding method as shown in Fig. 4.

activity was observed in all other tissues analyzed (data not shown). β-Hexosaminidase activity remained unchanged in the liver and fibroblasts from the mutant mice (data not shown).

Phenotypic Analysis of *α-Gal A(−/0)* Mice. To obtain hemizygous mice, heterozygous female mice, *α-Gal A(+/−)*, were mated with *α-Gal A(+/0)* male mice. Hemizygous male mice, *α-Gal A(−/0)*, were born in the expected ratio and exhibited clinically normal phenotype at 10–14 weeks of age. Histopathological analysis of 10-week-old mice revealed no obvious histological lesions in hematoxylin-eosin stained sections of kidney, liver, heart, spleen, lungs, and brain. Electron microscopy revealed lipid inclusions with electron-dense concentric lamellar structures in the lysosomes of renal tubular cells typical of those seen in patients with Fabry disease (Fig. 2). Other cellular components appeared morphologically normal. Using fluorescent-labeled *Griffonia (Bandeiraea) simplicifolia* lectin, which selectively binds to α-D-galactosyl residues, we analyzed kidneys of 10-week-old mice and embryonic fibroblasts by confocal microscopy. This analysis revealed intense fluorescence in the kidneys of the mutant mice indicating significant accumulations of compounds containing α-D-galactosyl residues (Fig. 3*C*). The specificity of this lectin binding was confirmed by competitive inhibition with galactose (Fig. 3*D*). Kidney sections from the littermate *α-Gal A(+/0)* mice did not exhibit similar intensity of staining (Fig. 3 *A* and *B*). Cultured fibroblasts from *α-Gal A(−/0)* mouse embryos also displayed significant accumulation of α-Gal A substrates, as reflected by the granular staining with intense fluorescence in the cytoplasmic compartment (Fig. 4*C*). Fibroblasts from the littermate controls did not stain (Fig. 4*A*). The specificity of the lectin binding in cultured fibroblasts was confirmed by competitive inhibition with galactose (Fig. 4 *B* and *D*). Analysis of neutral sphingolipids in liver and kidneys of *α-Gal A(−/0)* mice showed a striking accumulation of CTH (Fig. 5). Due to heterogeneity in the fatty acid moiety of this class of lipids in mice, two spots are characteristically observed for CTH on HPTLC.

Transduction of *α-Gal A*-Deficient Fibroblasts with Human α-Gal A. To correct the enzyme deficits in fibroblasts, we used two bicistronic retroviruses carrying both human *α-Gal A* cDNA and *MDR1* cDNA. The retroviruses, Ha-MDR-IRES-αGal and Ha-αGal-IRES-MDR, utilize an internal ribosome entry site (IRES; refs. 31 and 32) and generate a single transcribed mRNA of *α-Gal A* and *MDR1* in different order (11). The *α-Gal A*-deficient fibroblasts transduced with either retroviruses showed significant corrections in the enzyme deficits compared with mutant fibroblasts transduced with the control virus, HaMDR (Table 1). Transduction of *α-Gal A*-deficient fibroblasts with Ha-MDR-IRES-αGal retrovirus corrected the deficiency of enzymatic activity and caused the

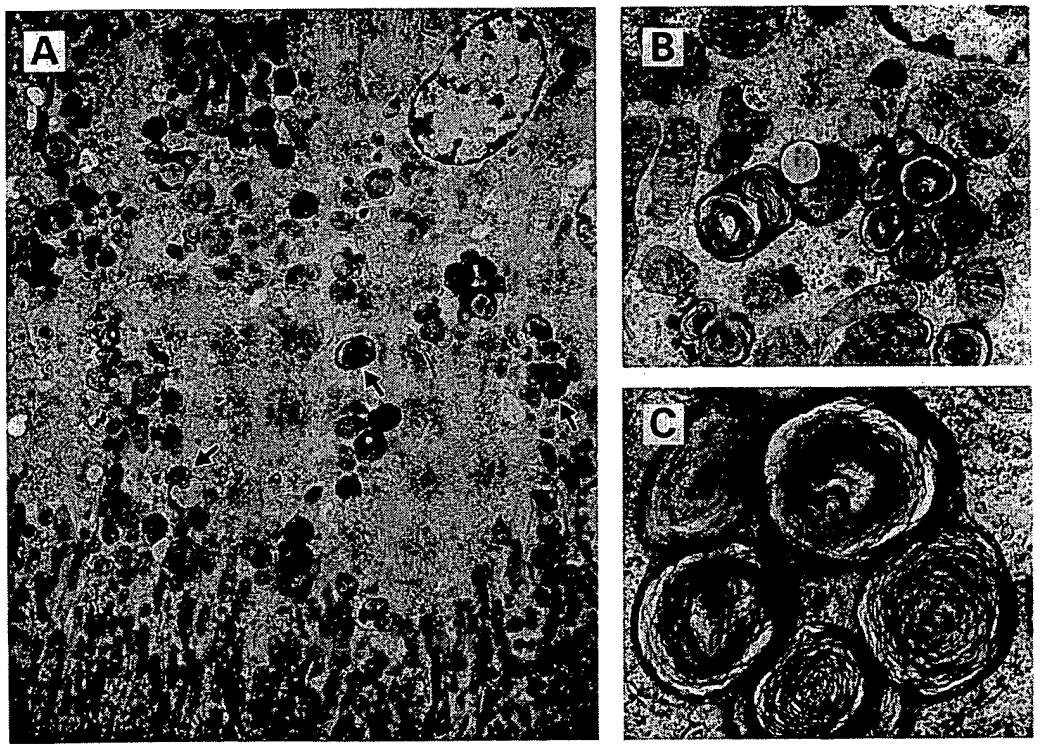

FIG. 2. Electron micrographs of kidneys from a 10-week-old *α-Gal A(−/0)* mouse. (*A*) Inclusions are seen in lysosomes of the renal tubular cells (arrows) (×6250). (*B*) Lysosomes contain inclusions of concentric lamellar structures (×20,000). (*C*) Higher magnification of inclusions in *B* (×60,000).

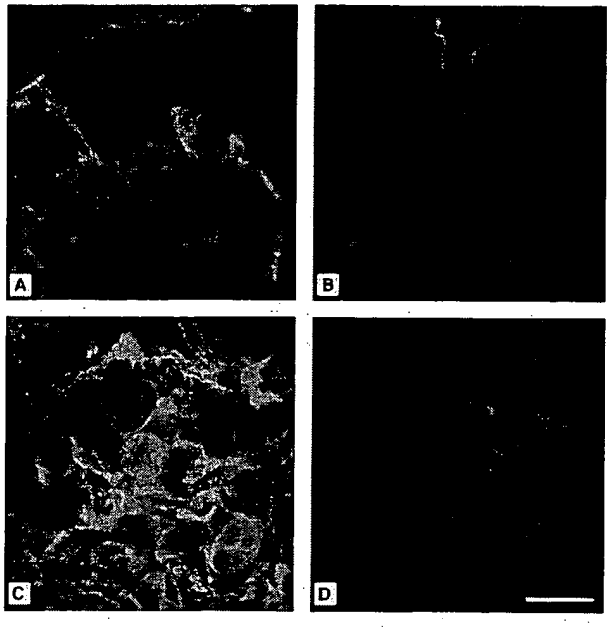

FIG. 3. FITC-labeled *Bandeiraea simplicifolia* lectin staining of kidneys from α-*Gal A(+/0)* (*A* and *B*) and α-*Gal A(−/0)* mice (*C* and *D*). (*A*) Kidney sections from a α-*Gal A(+/0)* mouse stained with FITC-labeled lectin. (*B*) Section as in *A* stained in the presence of 200 mM galactose. (*C*) Kidney section from a α-*Gal A (−/0)* mouse stained with FITC-labeled lectin. (*D*) Section as in *C* stained in the presence of 200 mM galactose. (Bar = 10 μm.)

clearance of accumulated material with α-galactosyl residues (Fig. 4*E*). Similar clearance was also observed in the mutant fibroblasts transduced with Ha-αGal-IRES-MDR retrovirus, but not in the fibroblasts transduced with HaMDR control (Table 1).

DISCUSSION

We have generated α-*Gal A*-deficient mouse lines by gene targeting. α-*Gal A*-deficient mice exhibit undetectable α-Gal A enzyme activity in the tissues and cultured fibroblasts, indicating a successful disruption of the α-*Gal A* gene. α-*Gal A*-deficient male and female mice appeared to be clinically normal at 10 weeks of age. However, ultrastructural analysis revealed typical lipid inclusions with lamellar structure in the lysosomes of renal tubular cells. Confocal microscopic analysis using *Griffonia (Bandeiraea) simplicifolia* lectin, which selectively binds to α-galactosyl residues, revealed intense staining in the kidneys and fibroblasts. Significant accumulation of CTH was observed in the liver and kidneys. Correction of the enzyme deficit and clearance of the accumulated residues occurred in the mutant fibroblasts that were transduced with MDR retroviruses containing human α-*Gal A* cDNA.

Electron microscopic analysis of 10-week-old mouse kidneys revealed inclusions typical of those seen in the human patients. Using specific lectin binding assay, we have demonstrated the accumulation of substrates with α-galactosyl residues in the kidneys as well as in the cultured fibroblasts. Most important, the HPTLC analysis revealed accumulation of CTH in the liver and kidneys. This reflects similarity in the pathophysiological events in α-*Gal A*-deficient mice and patients with Fabry disease. Although typical lamellar inclusions were seen in the lysosomes, other cellular components such as mitochondria and endoplasmic reticulum were well conserved in morphology. It is anticipated that as the α-*Gal A*-deficient mice age, CTH will continue to accumulate and that their phenotype will more closely resemble that of patients with Fabry disease in whom CTH has accumulated over decades. Early lethality of

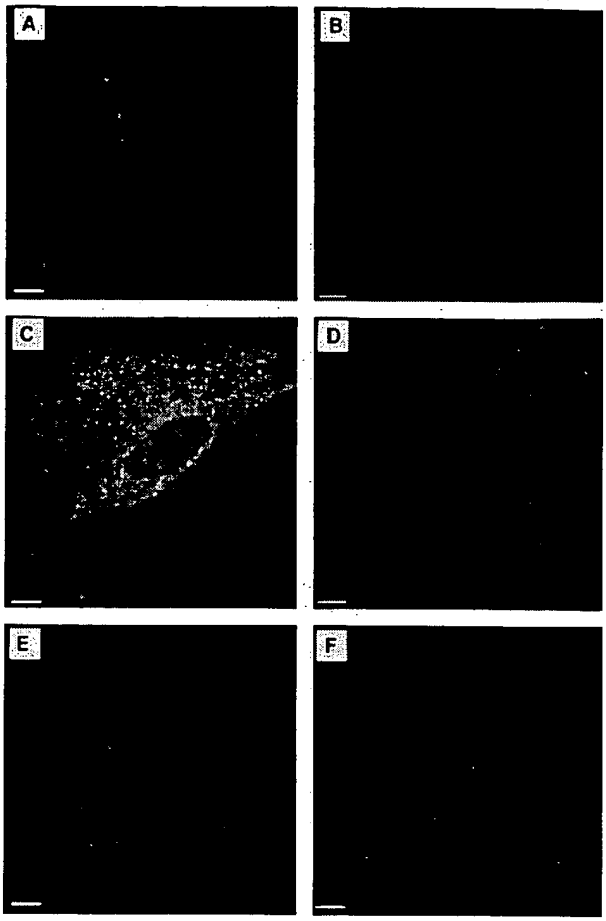

FIG. 4. FITC-labeled lectin staining of embryonic fibroblasts from α-*Gal A(+/0)* (*A* and *B*) and α-*Gal A(−/0)* (*C–F*) mice. Slides were incubated with lectin only (*A*, *C*, and *E*) or lectin and galactose (*B*, *D*, and *F*). (*D*) After transducing with MDR-based retrovirus (Ha-MDR-IRES-αGal), the KMG-2 clone showed the correction of metabolic deficit as seen by clearance of accumulated material (*E*). (Bars = 10 μm.)

mouse models for other lysosomal disorders pose difficulties for their usage in developing human therapy (33). Slow progression of disease process in α-*Gal A*-deficient mice offers a broad window for trial and evaluation of therapeutic strategies.

Gene therapy holds strong potential for the treatment of metabolic disorders (9, 10). However, this approach is still hampered by difficulties in obtaining sufficiently higher gene expression in target organs (34). In clinical trials, long-term transgene expression in hematopoietic cells has been disappointingly low (35). An alternate strategy for overcoming this problem is to employ the selectable drug-resistance markers that allow enrichment of transduced cells *in vivo*. *MDR1*, which

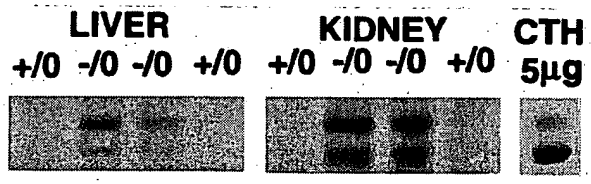

FIG. 5. Accumulation of CTH in liver and kidney from α-*Gal A(−/0)* mice. Liver and kidneys from wild-type (+/0, *n* = 2) and α-*Gal A*-deficient (−/0, *n* = 2) 10-week-old mice were analyzed by HPTLC as described. Purified porcine CTH (5 μg; Matreya, Pleasant Gap, PA) was used as a marker.

encodes *P*-glycoprotein, has been suggested as such a suitable gene for use in *in vivo* gene therapy (36). We have demonstrated the correction of enzyme deficits in the mutant cells transduced with the bicistronic MDR retroviruses containing human *α-Gal A* cDNA. Moreover, this enzyme correction leads to significant clearance of the accumulated substrates. Efficient expression of human *α-Gal A* using MDR retroviruses indicates the potential for the extension of these studies to *ex vivo* and *in vivo* gene therapy approaches in the *α-Gal A*-deficient mice.

In summary, *α-Gal A*-deficient mice generated by gene targeting are clinically normal at 10 weeks of age but exhibit accumulation of CTH and inclusions in the kidneys similar to those seen in Fabry patients. The correction of enzymatic and metabolic deficits in the *α-Gal A*-deficient fibroblasts transduced with MDR retroviruses containing human *α-Gal A* cDNA prompts detailed studies for *in vivo* correction of the *α-Gal A* deficiency. Experimental approaches to induce and treat the clinical phenotype in these mice should be valuable for the development of effective strategies for the treatment of patients with Fabry disease.

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